Three small RNAs jointly ensure secondary metabolism and biocontrol in *Pseudomonas fluorescens* CHA0

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In many Gram-negative bacteria, the GacS/GacA two-component system positively controls the expression of extracellular products or storage compounds. In the plant-beneficial rhizosphere bacterium *Pseudomonas fluorescens* CHA0, the GacS/GacA system is essential for the production of antibiotic compounds and hence for biological control of root-pathogenic fungi. The small (119-nt) RNA RsmX discovered in this study, together with RsmY and RsmZ, forms a triad of GacA-dependent small RNAs, which sequester the RNA-binding proteins RsmA and RsmE and thereby antagonize translational repression exerted by these proteins in strain CHA0. This small RNA triad was found to be both necessary and sufficient for posttranscriptional derepression of biocontrol factors and for protection of cucumber from *Pythium ultimum*. The same three small RNAs also positively regulated swarming motility and the synthesis of a quorum-sensing signal, which is unrelated to *N*-acylhomoserine lactones, and which autoinduces the Gac/Rsm cascade. Expression of RsmX and RsmY increased in parallel throughout cell growth, whereas RsmZ was produced during the late growth phase. This differential expression is assumed to facilitate fine tuning of GacS/A-controlled cell population density-dependent regulation in *P. fluorescens*.

GacA | posttranscriptional control

Bacteria react to changing environmental conditions and increasing cell population densities mostly by regulating the abundance of transcripts. In many signal transduction pathways, this occurs by activation or repression of mRNA synthesis (1). However, in an increasing number of cases studied, the primary transcripts that are environmentally or developmentally regulated turn out to be small noncoding RNAs. Many of these regulatory RNAs influence gene expression at a posttranscriptional level (2–5). For instance, in *Escherichia coli*, ≈20 different small RNAs have been shown to engage in base-pairing interactions with target mRNAs, resulting in activation or repression of translation and often in stabilization or destabilization of mRNAs (2–5). In *Vibrio cholerae*, four similar small RNAs (Qrr 1–4) are expressed at low cell population densities. The Qrr RNAs bind to and destabilize *hapR* mRNA, which encodes a major repressor of virulence genes. At high population densities, the Qrr RNAs are not expressed, and the corresponding stabilization of *hapR* mRNA leads to repression of virulence factors. Remarkably, deletion of all four Qrr RNAs is necessary to abolish this quorum-sensing control mechanism (6).

Bacterial small RNAs of another type act by sequestering RNA-binding proteins belonging to the CsrA (carbon storage regulator) family (7), which regulate translation initiation by binding to mRNA sequences near the ribosome-binding site. In *E. coli*, CsrA regulates the utilization of carbon sources, glycogen synthesis, biofilm formation, and motility (7–11), whereas in *Erwinia carotovora*, the CsrA homolog RsmA (repressor of secondary metabolism) controls the expression of extracellular enzymes and type III secretion (12). The similar RsmA and RsmE proteins of the plant-beneficial root-colonizing biocontrol strain CHA0 of *Pseudomonas fluorescens* negatively regulate the synthesis of extracellular antifungal secondary metabolites (13–15). Small noncoding RNAs, such as CsrB and CsrC of *E. coli* (8, 16), RsmB of *E. carotovora* (17), or RsmZ and RsmY of *P. fluorescens* (14, 18), bind multiple CsrA/RsmA molecules with high affinity and thereby allow translation of mRNAs, which are repressed by CsrA or RsmA. Among the factors that influence the expression of these small RNAs, the DNA-binding protein GacA stands out as a major activator (19, 20). The response regulator GacA is activated by phosphorylation from the cognate membrane-bond sensor GacS (21–24).

The GacS/GacA two-component system is conserved in many Gram-negative bacteria. Whereas in plant- and animal-pathogenic species GacS/GacA is important for virulence (20, 25–27), the same system is required for biocontrol in plant-beneficial strains (19, 20, 24, 28, 29). We have previously reported that, in *P. fluorescens* CHA0, GacS/GacA positively controls transcription initiation of two small RNA genes, *rsmZ* and *rsmY* (14, 18). However, RsmZ and RsmY alone cannot fully explain how the GacS/GacA system determines biocontrol activity, because an *rsmY rsmZ* double mutant retains partial expression of biocontrol traits (18). Here, we report the discovery of a third GacA-controlled small RNA, RsmX, in *P. fluorescens* and show that the simultaneous absence of RsmX, RsmY, and RsmZ RNAs mimicks the biocontrol-negative phenotype of *gacS* and *gacA* mutants. This triad of small RNAs was also found to control swarming motility and the synthesis of a low-molecular-weight quorum-sensing signal that induces the Gac/Rsm cascade.

Materials and Methods

Bacterial Strains and Culture Conditions. *P. fluorescens* CHA0 (wild type), CHA19 (Δ*gacS*), CHA89 (*gacA*::Km), CHA810 (Δ*rsmZ*), CHA822 (Δ*rsmY*), CHA825 (Δ*rsmY* Δ*rsmZ*), CHA1003 (*rsmE*::Ω-Hg), CHA1008 (Δ*gacS rsmE*::Ω-Hg *rsmA*::Ω-Km), CHA1009 (*rsmE*::Ω-Hg *rsmA*::Ω-Km), and CHA1076 (*rsmA*::Ω-Km) and their derivatives carrying chromosomal *hcnA′-′lacZ* or *aprA′-′lacZ* fusions have been described (14, 15, 18, 19, 24). Strains carrying a chromosomal *rsmE′-′lacZ* fusion were constructed by using pME7545 for delivery (29). Growth conditions, antibiotic concentrations, and conditions for β-galactosidase assays were the same as those previously used (14, 15, 18).

RNA Extraction and Northern Blot Analysis. These were performed as described (14, 18). Hybridizations were done with a digoxigenin-labeled DNA probe generated by PCR covering the entire

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Abbreviations: Csr, carbon storage regulator; Rsm, repressor of secondary metabolism.

Data deposition: The *rsmX* sequence reported in this paper has been deposited in the GenBank database (accession no. DQ137846).

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rsmX structural gene with primers PTX3hind and PTX4eco (Table 3, which is published as supporting information on the PNAS web site).

Isolation of *rsmX*. His-tagged RsmA and RsmE (RsmA6H and RsmE6H) were overexpressed in strain CHA825 carrying pME6078 or pME7011 and purified by Ni-nitrilotriacetic acid affinity chromatography, as described (14). RNA was separated from the purified proteins by two extractions with phenol/chloroform (1:1) and once with chloroform. RNA was ethanol-precipitated, dissolved in diethylpyrocarbonate-treated water, and electrophoresed on a denaturing urea-polyacrylamide gel. An RNA band of ≈100 nucleotides was extracted with the ElutaTube RNA Extraction Kit (Fermentas, Hanover, MD). cDNA synthesis was performed as described (8) by using random hexameric primers and the Universal Riboclone cDNA Synthesis System (Promega). The resulting blunt-ended cDNA fragments were cloned into SmaI-digested phosphatase-treated pBluescript KS (Stratagene). Ten independent clones were sequenced by using primers T3 and T7; the inserts of two clones contained an *rsmX* fragment. The *rsmX* gene was located between the genes PFL-4113 and PFL-4112 on the chromosome of *P. fluorescens* Pf-5 (ref. 30; www.tigr.org) by using the program FUZZNUC (http://bioweb.pasteur.fr/seqanal/interfaces/fuzznuc.html).

The *rsmX* gene and flanking sequences were obtained by PCR amplification of *P. fluorescens* CHA0 chromosomal DNA with primers PRX1hind and PRX2eco (Table 3), which were designed according to the genome sequence of *P. fluorescens* Pf-5. The 1.6-kb PCR product was cloned into pBluescript KS (giving pME7312) and sequenced. A 64-bp deletion in the chromosomal *rsmX* gene (extending from nucleotide + 17 to nucleotide + 81) was constructed as follows. Two sequences flanking *rsmX* were PCR-amplified from CHA0 chromosomal DNA with primers PRX1hind + PMUBAM2 and PRX2eco + PMUBAM1 (Table 3). The resulting 635-bp upstream and 955-bp downstream fragments were cleaved with HindIII + BamHI, and BamHI + EcoRI, respectively, and inserted into the suicide plasmid pME3087 (14) cut with HindIII + EcoRI. The resulting plasmid pME7315 was introduced into strains CHA0, CHA207, CHA805, CHA825, CHA826, and CHA827 (13, 14, 18) to generate, via homologous recombination, the corresponding mutants CHA1141 (Δ*rsmX*), CHA1142 (Δ*rsmX*, *hcnA'-'lacZ*), CHA1143 (Δ*rsmX*, *aprA'-'lacZ*), CHA1144 (Δ*rsmX* Δ*rsmY* Δ*rsmZ*), CHA1145 (Δ*rsmX* Δ*rsmY* Δ*rsmZ*, *hcnA'-'lacZ*), and CHA1146 (Δ*rsmX* Δ*rsmY* Δ*rsmZ*, *aprA'-'lacZ*). An *rsmX-lacZ* fusion was constructed by PCR amplification of the 312-bp *rsmX* promoter region with primers PROF and PROR1 (Table 3). The product was digested by EcoRI and BamHI and introduced into pME6916 (18) digested with the same enzymes to produce pME7317, in which the +1 of the *lacZ* gene is fused to the +4 site of the *rsmX* promoter. An *rsmX* overexpression plasmid was constructed as follows. A PCR product containing *rsmX* was obtained with pME7312 and primers PTX4eco + POVEX (Table 3), digested with EcoRI, and cloned into pME6552 [=pUK21-Ptac (13)] cut with KpnI (blunted) and EcoRI, resulting in pME7319. A 0.9-kb MluI-EcoRI fragment of pME7319 carrying the *tac* promoter fused at the +1 site to *rsmX* was inserted into pME6032 (14), producing pME7320. This construct was introduced into strains CHA89–207 (13), CHA806 (14), CHA1145, and CHA1146.

***In Vitro* Transcription of *rsmX* and Gel Mobility-Shift Assays.** The *rsmX* gene was PCR-amplified with primers PTZhind and PTZeco (Table 3) and cloned under the control of the T7 promoter in pTZ19R (Fermentas) to produce pME7318. The radioactively labeled RsmX transcript was synthesized from linearized pME7318 with the T7 transcription kit (Fermentas) in the presence of [α-^{33}P]UTP and purified (18). RsmA6H and RsmE6H were overexpressed in *E. coli* DH5α/pME6078 and BL21/pME7013, respectively; purified; dialyzed against 10 mM Tris-acetate, pH 7.5, at 4°C; concentrated on a Centricon membrane (Millipore); and stored at −20°C. Binding reactions were carried out with labeled RsmX and RsmA6H or RsmE6H, as described (15, 18).

Detection of Exoproducts and Swarming Motility. Hydrogen cyanide production was measured as described (31). Exoprotease activity was detected on skim-milk agar (0.4% blood agar base/0.05% yeast extract/15% skim milk/1.35% agar) by spotting 10^7 cells in 10 μl of 0.9% NaCl. 2,4-Diacetylphloroglucinol was extracted with ethyl acetate from cell cultures grown to OD_{600} ≈7 in 100 ml of a medium containing 0.75% malt extract, 1% proteose peptone, 0.5% glycerol, 0.075% $MgSO_4$, and 0.075% K_2HPO_4; incubation was for 40 h. The extract was dried under vacuum, dissolved in 200 μl of methanol, and analyzed by HPLC, as described (32). The quorum-sensing signal was extracted with dichloromethane from culture supernatants of strains CHA0, CHA89, and CHA1144, as described (14). For semiquantitative

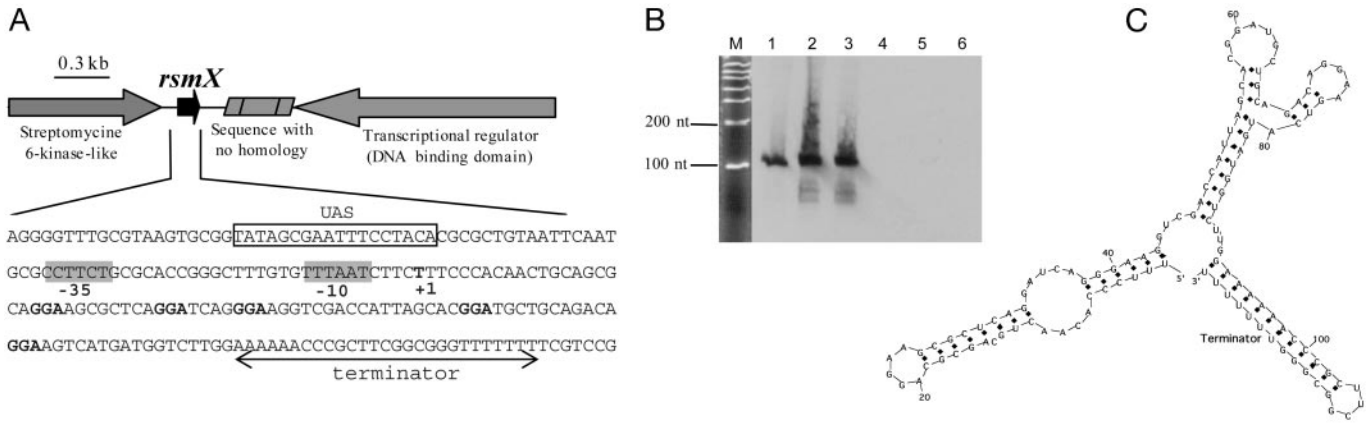

Fig. 1. The *rsmX* gene is not linked to *gacS* and *gacA*, depends on *gacA* function for expression, and shows characteristic unpaired GGA motifs. (*A*) Organization of the *rsmX* region of *P. fluorescens* CHA0. The −35 and −10 promoter sites are indicated with gray boxes. The palindromic sequence boxed from −70 to −53 denotes the upstream activating sequence (UAS), the putative GacA regulatory site. A sequence of 311 nucleotides (rhomboid box), which is absent from strain Pf-5, does not present any homology with a known sequence. (*B*) Northern blot showing the differential temporal accumulation of RsmX. Total RNA was extracted from CHA0 (wild type; lanes 1–3), CHA89 (*gacA*; lanes 4–6). OD_{600} values at the time of harvesting were, in lanes 1 and 4, 0.5; in lanes 2 and 5, 1.9; and in lanes 3 and 6, 2.5. (*C*) Predicted secondary structure of RsmX at 30°C using the MFOLD program (www.bioinfo.rpi.edu/applications/mfold).

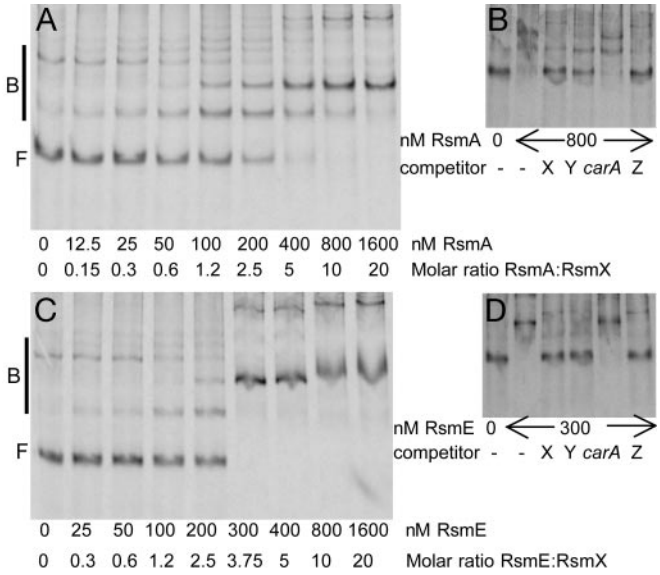

Fig. 2. RsmA and RsmE bind to RsmX, as demonstrated by RNA gel mobility-shift analysis. RsmX was synthesized *in vitro* by T7 RNA polymerase in the presence of [α-^{33}P]-UTP. Labeled RsmX (80 nM) was incubated with the concentrations of RsmA or RsmE indicated in *A* and *C*, respectively. The positions of free (F) and bound (B) RNA species are indicated. Unlabeled competitor RNAs (X = RsmX, Y = RsmY, and Z = RsmZ and CarA leader) were synthesized following the same protocol but with unlabeled UTP. Labeled RsmX (80 nM) with either specific (RsmX, RsmY, or RsmZ) or nonspecific (CarA leader) nonlabeled competitors (300 nM) was incubated with 800 nM RsmA and 300 nM RsmE in *B* and *D*, respectively.

determination of signal molecules produced by these strains, different amounts of extracted supernatants were added to the reporter strain CHA0/pME6530 (*hcnA′-′lacZ*) (13). The amount of culture extract resulting in half-maximal induction of the reporter gene was calculated from Hanes plots ($[S]\ v^{-1} = V_{max}{}^{-1}\ [S] + K_m\ V_{max}{}^{-1}$, where $[S]$ is the amount of extract, v is the β-galactosidase-specific activity of the reporter, V_{max} is maximal specific activity, and K_m the amount of extract giving half-maximal induction). Swarming motility was assessed on semisolid medium (0.8% nutrient broth/0.5% glucose/0.5% agar) (33).

Biocontrol Assay in Natural Soil. Ten flasks containing three plants each were planted with cucumber and treated with *Pythium ultimum* and *P. fluorescens*, as described (18).

Results

Discovery of the GacA-Controlled Small RNA RsmX. In *P. fluorescens* CHA0, the RNA-binding protein RsmA, together with its homolog RsmE, represses translation of biocontrol genes in the Gac/Rsm cascade (15). Histidine-tagged RsmA and RsmE were separately overexpressed as a bait in the *rsmY rsmZ* double mutant CHA825, purified by affinity chromatography, and stripped of RNA molecules, which were converted to cDNA clones (see *Materials and Methods*). Among these cDNAs, two fragments belonged to a small RNA gene that was termed *rsmX*. Based on the genomic sequence of *P. fluorescens* Pf-5, which is closely related to strain CHA0 (30, 34), primers were designed to amplify the entire *rsmX* gene, including flanking sequences. The 1.6-kb *rsmX* region thus cloned was 99% identical to that of strain Pf-5, except for an insert of 311 nucleotides, which occurred only in the CHA0 chromosome downstream of *rsmX* (Fig. 1*A*). Expression of RsmX increased with increasing cell population densities in the wild type and totally depended on GacA function (Fig. 1*B*). The *rsmX* transcription start was mapped by RACE (described in *Supporting Text* and Fig. 6, which are published as supporting information on the PNAS web site). The deduced RsmX RNA (≈119 nt) is predicted to have an elaborate secondary structure with a ρ-independent terminator and four potentially unpaired GGA motifs (Fig. 1*C*). These motifs are characteristic of RNAs that bind CsrA/RsmA-type proteins (7, 8, 11, 16–18). Homologs of *rsmX* were found in the database for *P. fluorescens* strains Pf-5 and Pf0–1, as well as for *P. syringae* pv. *tomato* DC3000, but not for *P. aeruginosa* (data not shown).

RsmX Specifically Binds RsmA and RsmE Proteins. *In vitro*, a 4- to 5-fold molar excess of RsmA (Fig. 2*A*) or RsmE (Fig. 2*C*) over RsmX completely converted the free RNA molecule to complexes of higher molecular weights. The specificity of the RsmX–RsmA (Fig. 2*B*) and RsmX–RsmE (Fig. 2*D*) interactions was verified: unlabeled RsmX, RsmY, or RsmZ competed effectively for the RNA-binding proteins, whereas an unrelated mRNA fragment (*carA*) did not. From these and published observations (15, 18), we conclude that RsmX, RsmY, and RsmZ all have similar high affinities for RsmA and RsmE.

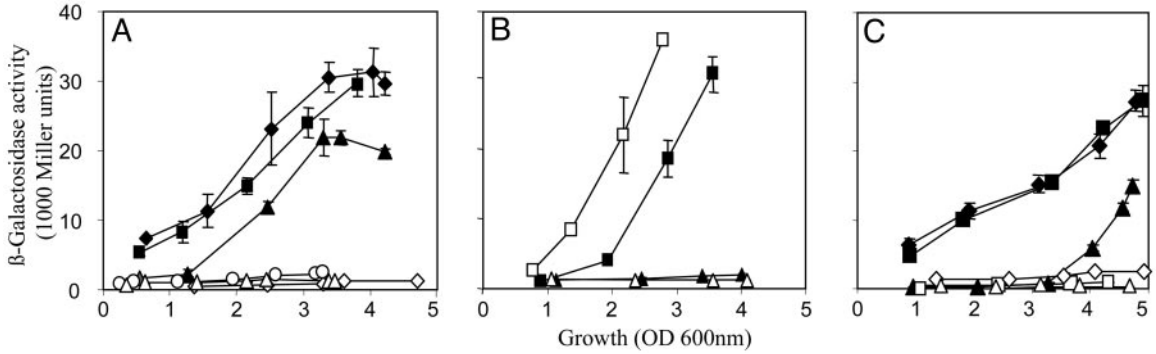

Fig. 3. The expression of the *rsmX* gene is controlled by GacA, RsmA, RsmE, and the CHA0 signal in *P. fluorescens* CHA0. (*A*) Activation of *rsmX* expression by RsmA and RsmE. β-Galactosidase activities of a transcriptional *rsmX-lacZ* fusion carried by pME7317 were determined in the wild type (CHA0; squares), a *gacA* mutant (CHA89; open diamonds), an *rsmE* mutant (CHA1003; diamonds), an *rsmA* mutant (CHA1076; triangles), an *rsmAE* double mutant (CHA1009; open circles), and a *gacS rsmAE* mutant (CHA1008; open triangles). (*B*) Activation of *rsmX* expression by the signal from CHA0. β-Galactosidase activities of an *rsmX-lacZ* fusion were determined in the wild-type strain without (squares) or with extract (open squares) and the *gacA* mutant without (triangles) or with extract (open triangles). (*C*) Differential temporal expression of *rsmX*, *rsmY*, and *rsmZ*. β-Galactosidase activities of the transcriptional *rsmX-lacZ* (pME7317; squares), *rsmY-lacZ* [pME6916 (18), diamonds], and *rsmZ-lacZ* [pME6091 (14), triangles] were determined in strain CHA0 (wild-type; filled symbols) and in the *gacA* mutant CHA89 (empty symbols).

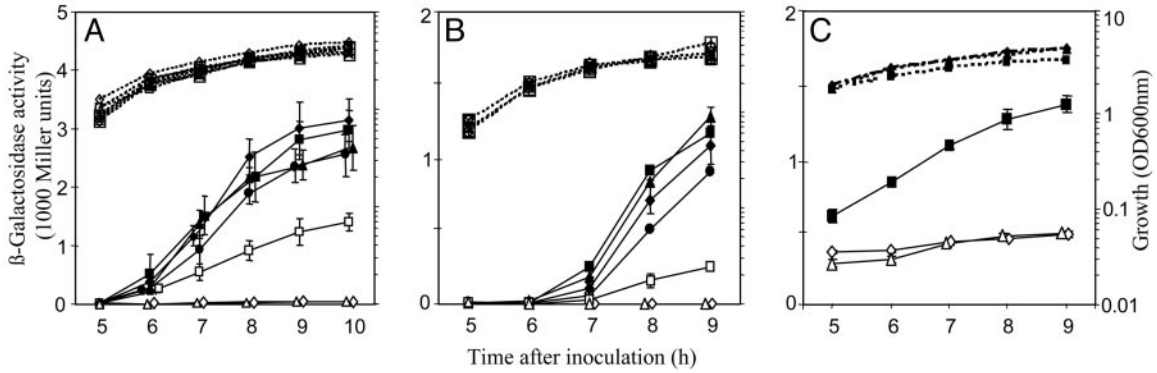

Fig. 4. Deletion of *rsmX*, *rsmY*, and *rsmZ* results in low expression of target genes similar to that found in a *gacA* mutant. (*A*) Expression of a chromosomal *hcnA′-′lacZ* translational fusion and growth of the wild type (CHA207; squares), a *gacA* mutant (CHA89.207; open diamonds), an *rsmX* mutant (CHA1142; diamonds), an *rsmY* mutant (CHA823; circles), an *rsmZ* mutant (CHA811; triangles), an *rsmY rsmZ* mutant (CHA826; open squares), and an *rsmX rsmY rsmZ* mutant (CHA1145; open triangles). (*B*) Expression of a chromosomal *aprA′-′lacZ* translational fusion and growth in the wild-type (CHA805; squares), a *gacS* mutant (CHA806; open diamonds), an *rsmX* mutant (CHA1143; diamonds), an *rsmY* mutant (CHA824; circles), an *rsmZ* mutant (CHA812; triangles), an *rsmY rsmZ* mutant (CHA827; open squares), and an *rsmX rsmY rsmZ* mutant (CHA1146; open triangles). (*C*) Expression of a chromosomal *rsmE′-′lacZ* fusion in the wild type (CHA1134; squares), a *gacA* mutant (CHA1136; diamonds), and an *rsmX rsmY rsmZ* mutant (CHA1164; triangles). Growth is indicated by dotted lines.

Regulation of *rsmX*. The *rsmX* promoter was fused, at the fourth nucleotide downstream of the transcription start site, to a transcriptional *lacZ* reporter in pME7317. This construct gave increasing β-galactosidase expression with increasing cell population densities (Fig. 3*A*). Like *rsmY* and *rsmZ* (14, 15, 18), *rsmX* was silent in a *gacA* mutant and very poorly expressed in an *rsmA rsmE* double mutant; in *rsmA* or *rsmE* single mutants, *rsmX* was transcribed almost normally (Fig. 3*A*). A crude preparation of a low-molecular-weight quorum-sensing signal, which is produced by strain CHA0 in late exponential phase and which induces the expression of *rsmY* (18) and *rsmZ* (14), also induced *rsmX* transcription ≈3-fold in strain CHA0 (Fig. 3*B*). By contrast, in a *gacA* (Fig. 3*B*) or *rsmA rsmE* mutant (data not shown), no induction was observed, suggesting that GacA, RsmA, and RsmE together are involved in the transduction of this signal [which are unrelated to *N*-acyl-homoserine lactones (24) and has not yet been identified chemically]. GacA-dependent expression of *rsmX-lacZ* and *rsmY-lacZ* occurred in parallel during the entire growth cycle, whereas *rsmZ-lacZ* expression was delayed (Fig. 3*C*).

Regulation of Target Genes in the Gac/Rsm Cascade. An *rsmX rsmY rsmZ* triple mutant, CHA1144, was constructed, by introducing a 64-nt deletion (Fig. 1*A*) into the *rsmX* gene in the Δ*rsmY* Δ*rsmZ* mutant CHA825 (*Materials and Methods*). *In vitro* growth of strain CHA1144 was indistinguishable from that of the wild-type CHA0 (Fig. 4*A*). Strain CHA1144 and the *gacA* mutant CHA89 showed equally low basal expression levels of typical biocontrol genes, *hcnA* and *aprA* (Fig. 4 *A* and *B*), which are needed for the production of hydrogen cyanide (HCN) and the major exoprotease, respectively (35, 36). Similarly, both strains CHA1144 and CHA89 gave strongly reduced expression of the *rsmE* gene, by comparison with wild-type CHA0 (Fig. 4*C*). This finding was confirmed by monitoring the RsmE protein by Western blotting; in the *rsmX rsmY rsmZ* triple mutant, RsmE levels were as strongly decreased (Fig. 7, which is published as supporting information on the PNAS web site, and *Supporting Text*), as they are in a *gacA* mutant (15). In the same experiment, we verified that the RsmA protein levels were not influenced by *rsmX rsmY rsmZ* expression. We also measured the GacA-controlled extracellular products HCN, AprA exoprotease, and 2,4-diacetylphloroglucinol (32) in culture supernatants; both strains CHA89 and CHA1144 were equally deficient, whereas mutations in *rsmX* alone or *rsmY rsmZ* had less drastic consequences (Table 1). Swarming motility, which depends on GacA (37), presented an analogous pattern in the same set of strains (Table 1). Interestingly, strain CHA0 produced ≈25 times higher amounts of the inducing signal than did the *gacA* and *rsmX rsmY rsmZ* mutants (Table 1). In conclusion, these results indicate that RsmX, RsmY, and RsmZ together are needed to control the

Table 1. Biosynthesis of exoproducts and swarming in *P. fluorescens* CHA0 and *gacA, rsmX, rsmYZ,* and *rsmXYZ* mutants

Strain	Genotype	HCN* (nmol/10^9 cells)	DAPG† (nmol/10^9 cells)	Exoprotease activity‡	Inducing signal,§ %	Swarming motility¶
CHA0	Wild type	277 ± 86	58.4 ± 3.9	++	100	+++
CHA89	*gacA*::Km^r	5.3 ± 1.3	<1.0	−	4.3 ± 1.3	−
CHA1141	Δ*rsmX*	360 ± 12	58.7 ± 2.4	++	ND	++
CHA825	Δ*rsmYZ*	76 ± 37	<1.0	+	ND	+
CHA1144	Δ*rsmXYZ*	4.6 ± 0.6	<1.0	−	4.1 ± 0.9	−

*Average values of three measurements ± SD.
†Average values of six independent cultures ± SD.
‡Halo of 5 mm (++), 2 mm (+), or no halo (−) after 24 h of growth at 30°C.
§The relative amount of inducing signal was estimated from the culture volume required to give half-maximal induction of the *hcnA′-′lacZ* fusion on pME6530 (13). One hundred percent corresponds to 2.9 ml of CHA0 culture volume. ND, not done.
¶Ability to swarm on semisolid medium after 72 h of incubation at room temperature: 30 mm (+++), 20 mm (++), 7 mm (+), no swarming (−).

Table 2. A *P. fluorescens rsmX rsmY rsmZ* triple mutant does not protect cucumber seedlings from *Pythium* damping-off and root rot

Strain added	Genotype	*Pythium* added	Number of emerged plants per flask* (%)	Shoot fresh weight per flask* (g)	Root fresh weight per flask* (g)	*P. fluorescens*† ($\log_{10}$ colony-forming units per gram of roots)
None		−	100 a	1.17 a, b	0.41 a	−
CHA0	Wild type	−	100 a	1.34 a	0.44 a	6.3
CHA19	Δ*gacS*	−	100 a	1.35 a	0.47 a	4.6
CHA1144	Δ*rsmXYZ*	−	100 a	1.27 a	0.42 a	4.9
None		+	83 a, b	0.72 c	0.24 b	−
CHA0	Wild type	+	96 a	1.11 a, b	0.45 a	6.6
CHA19	Δ*gacS*	+	86 a, b	0.86 b, c	0.32 a,b	5.6
CHA1144	Δ*rsmXYZ*	+	76 b	0.66 c	0.25 b	5.3

*Data represent the averages of 10 replicates (flasks containing three cucumber plants) per treatment. Means within the same column followed by different letters differ significantly at $P = 0.05$, according to Tukey's honestly significant difference post-hoc test.

†*P. fluorescens* strains carrying the stable plasmid pME6010 (Tc^R) were to determine colonization ability.

expression of target genes in the Gac/Rsm cascade, and that the inducing signal is produced as an integral part of the cascade.

Overexpression of *rsmX* Can Compensate for Lack of *rsmY* and *rsmZ*. It has been shown that artificial overexpression of *rsmY* or *rsmZ* suppresses the negative effects of *gacS* and *gacA* mutations on target gene expression (14, 18, 38). When *rsmX* was overexpressed from the *tac* promoter (on pME7320) in an *rsmX rsmY rsmZ* mutant background, the expression of an *hcnA′-′lacZ* fusion was restored to 170%, and that of an *aprA′-′lacZ* fusion to 30%, relative to the expression of these fusions in the wild type (set at 100%). Similarly, overexpression of *rsmX* suppressed *gacS* and *gacA* mutations in *P. fluorescens* (data not shown). These results indicate that RsmX, RsmY, and RsmZ can functionally replace each other to a considerable extent. Whether these small RNAs display some target specificity is still a matter of speculation (39).

An *rsmX rsmY rsmZ* Mutant Lacks Biocontrol Activity. Strain CHA0 protects cucumber from disease caused by the oomycete *P. ultimum* in natural soil (18). The *rsmX rsmY rsmZ* mutant was devoid of such biocontrol activity (in terms of emerged plants and plant fresh weight) and was slightly impaired in root colonization in natural soil. The properties of a *gacS* mutant were not significantly different in this experiment (Table 2). We conclude that the three small RNAs, RsmX, RsmY, and RsmZ, together are required for biocontrol activity of *P. fluorescens* CHA0.

Discussion

Three Small RNAs Jointly Execute Posttranscriptional Regulation of Biocontrol Factor Synthesis. Mutation of either *gacS* or *gacA* results in a 30- to 100-fold reduction of biocontrol factor expression in *P. fluorescens* CHA0 (13, 19, 24). This strong global effect on genes involved in exoproduct synthesis occurs essentially at a posttranscriptional level and is superimposed on and epistatic over various types of transcriptional control of individual target genes (40). As we have shown here, a triple *rsmX rsmY rsmZ* mutation was needed to cause the same phenotypic effects on exoproduct formation and biocontrol as those observed in *gacS* or *gacA* mutants. It therefore appears unlikely that additional small RNAs would participate in the activation of exoproduct synthesis via the Gac/Rsm cascade. However, this does not exclude the possibility that GacS/GacA might control the expression of additional small RNAs having different functions.

Potential Benefits of Triple RNA Control. RsmX, RsmY, and RsmZ of *P. fluorescens* have similar affinities for RsmA and RsmE (ref. 15; Fig. 2) and, in this respect, they appear to be functionally redundant. By contrast, in plant-pathogenic *Erwinia* spp., there appears to be only one GacA-controlled regulatory RNA termed RsmB, which has 479 nucleotides, thus about the size of RsmX, RsmY, and RsmZ together (17). The Gac/Rsm cascade controls the expression of pathogenicity factors in *Erwinia* spp.; both *gacA* and *rsmB* mutants are less virulent (17, 41). In *E. coli*, two GacA(=UvrY)-dependent small RNAs, CsrB and CsrC, are needed for regulation of central carbon metabolism and carbon storage (7, 11, 16). Only one RNA-binding protein (RsmA/CsrA) is sequestered by these small RNAs in *Erwinia* spp. as well as in *E. coli*. The question then arises what benefits *P. fluorescens* may obtain from having three GacA-controlled small RNAs (RsmX, RsmY, and RsmZ) as antagonists of two RNA-binding proteins (RsmA and RsmE). In principle, this arrangement may allow a more efficient regulatory response via a gene dosage effect. However, when any one of the three small RNA genes is deleted, the phenotypic consequences are minor (Table 1; Fig. 4 *A* and *B*; refs. 14 and 18). In addition, overexpression of

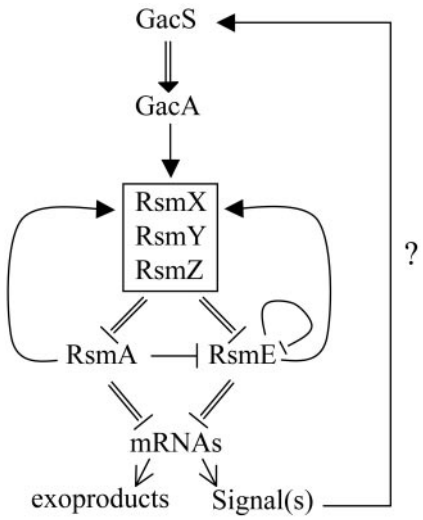

Fig. 5. Model for gene regulation in the Gac/Rsm system of *P. fluorescens*. —, direct or indirect regulation; =, physical interaction; →, positive effect; ⊣, negative effect. A physical interaction of the signal with GacS has not been demonstrated experimentally.

rsmX, *rsmY*, or *rsmZ* alone from a strong vector promoter to a large extent overrides the negative effects of *gacS* and *gacA* mutations. Alternatively, differential expression of the *rsmX*, *rsmY*, and *rsmZ* genes may be advantageous, because it may allow fine tuning of the Gac/Rsm system in response to different environmental stimuli. Whereas *rsmX* and *rsmY* were expressed similarly under our *in vitro* conditions, expression of *rsmZ* was clearly distinct (Fig. 3*C*). Functional redundancy of small RNAs has also been observed in *P. aeruginosa*, where two Fur-controlled RNAs, PrrF1 and PrrF2, regulate the expression of genes involved in iron storage and resistance to oxidative stress. By contrast, the same function is carried out by a single homolog, RyhB, in *E. coli* (42).

Signaling Creates a Positive Feedback Loop in the Gac/Rsm Cascade of *P. fluorescens*. This study provides evidence for an important feedback mechanism operating in the Gac/Rsm signal transduction pathway of *P. fluorescens*. The synthesis of the signal molecules that lead to activation of target gene expression in the Gac/Rsm cascade (14, 24) depends on GacA and on the three small RNAs (Table 1). The signal activates transcription of *rsmX* (Fig. 3*B*), *rsmY* (18), and *rsmZ* (14). Perception of the signal needs functional GacS (24). Biocontrol factors and signal are produced in parallel at the end of exponential growth (data not shown). By this mechanism, the Gac/Rsm cascade (summarized in Fig. 5) positively autoregulates its activity as a function of increasing cell population densities. Similar autoinduction patterns are common in quorum-sensing regulation depending on *N*-acyl-homoserine lactone signaling (43). As *Vibrio* spp. (6), *P. fluorescens* CHA0 has a set of apparently redundant small RNAs that have a key function in cell–cell communication and in the regulation of extracellular products.

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